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Carbon dots enhanced cold tolerance of lettuce (*Lactuca sativa* L): scavenging reactive oxygen species, modulating hormones and up-regulating gene expression

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Supplementary Text S1: SOD, CAT and MDA testing

Referring to the previous scheme, follow the steps below to conduct the experiment.[1] Preparation of enzyme solution: Fresh lettuce leaves (0.2 g) and 0.05 mol·L^{-1} phosphate buffered saline (PBS) (2 ml) at pH 7.8 were mixed together for ice-bath grinding. The mixture was transferred to a centrifuge tube, and centrifuged at 4℃ and 12000 g for 20 min. The supernatant in the centrifuge tube was the enzyme solution.

SOD determination: The components of the reaction solution are as follows: 162 ml of 14.5 uM methionine (Met) solution, 0.6 ml of 30 uM EDAT−Na2 solution, 6 ml of 0.05 mol·L^{-1} PBS with pH 7.8, 6 ml of 2.25 mM nitroblue tetra azole (NBT) and 6 ml of 60 uM riboflavin. The above components are mixed to form the reaction solution. 3ml of the reaction solution and 30 μl of the enzyme solution were mixed and reacted under 4000 lux light conditions for 20 min. After the reaction, the absorbance was measured at 560 nm under the dark condition. The determination of SOD needs to prepare two sets of controls. The control group 1 is the maximum photoreduction tube, 3ml of reaction solution and 30 ul of PBS are mixed, and the steps are the same as before. The control group 2 was composed of PBS buffer, and was directly measured for zero adjustment under light-shielded conditions.

CAT determination: The reaction solution is composed of 200 ml of 0.15 mol·L^{-1} pH7.0 PBS and 0.3092 ml of 30% H_2O_2. The test of CAT activity requires 3 ml of reaction solution and 0.1 ml of enzyme solution, and the absorbance is measured at 240 nm after mixing. Zeroing is done with PBS.

MDA determination: 0.5 g of fresh lettuce leaves and 2 ml of 0.05 mol·L^{-1} PBS with pH 7.8 were mixed, ground in an ice bath, centrifuged at 4500 r/min for 10 min, and the supernatant obtained by centrifugation was the MDA extract. Take 2 ml of malondialdehyde extract, add 3ml of trichloroacetic acid containing 0.5% thiobarbituric acid (TBA), heat in a boiling water bath for 10 min, cool down rapidly and centrifuge at 4500 r/min for 10 min, take the supernatant to measure the absorbance. Absorbance was measured at 532 and 600 nm, and distilled water was used as a blank.

Supplementary Text S2: Quantitative real-time PCR

Total RNA was extracted using RNApure Plant Kit (DNase I) (CWBIO, China), and

the extracted RNA was reverse transcribed using EasyQuick RT MasterMix (CWBIO, China) to obtain cDNA. Real-time PCR detection system using CFX 96 Optical Module (BioRad) with UltraSYBR Mixture (CWBIO, China) for quantitative representation of target genes. Details of the reference genes and primers are listed in the Table S3.[3]

Supplementary Text S3: HPLC–MS analysis of lettuce leaves

The supernatant was spun dry and re-dissolved in 200 μL of methanol acetonitrile water (4:4:2), and the supernatant was removed by centrifugation at 4 ℃ and 12000 rpm for 15 min after 30 min of ultrasonication in an ice bath (35 kHz). The supernatant was removed by centrifugation at 4 ℃ and 12000 rpm for 10 min. Quality control (QC) samples were prepared by mixing aliquots of all samples (15 μL of each methanolic extract). Blanks were set to 80% methanol solution. The samples were analyzed by high performance liquid chromatography-tandem mass spectrometry (HPLC–MS, APS8016PLUS, Thermo Scientific, Germany) with reference to the previous method.[4] The test results were analyzed by MetaboAnalyst 5.0.

Parameters set of HPLC-MS

Liquid phase conditions

1) Chromatographic column: HSS T3 column (100*1.7 mm, particle size 1.8 μm, Waters); 2) Column temperature: 35°C; 3) Mobile phase: A = 0.1% formic acid/water; B = 0.1% formic acid/ Acetonitrile; 4) Elution gradient: 0 min 5% B; 1.5 min 5% B; 10 min 100% B; 11 min 100% B; 11.5 min 5% B; 14 min 5% B; 5) Injection volume: 5 μL; 6) Flow rate: 0.35 mL/min.

Mass spectrometry conditions

1) General: Runtime 0–14 min; Polarity negative/positive; Default charge 1; 2) Full MS: Resolution 70000; AGC target 1e6; Maximum IT 100 ms; Scan range 70−1050 m/z; 3) dd−MS^2 /dd−SIM: Resolution 17500; AGC target 5e4; Maximum IT 50 ms; Loop count 8; TopN 8; Isolation window 1.5 m/z; (N)CE/Stepped nce nce: 20, 40, 60; 4) dd Settings : Minimum AGC target 8e3; Intensity threshold 1.6e5; Exclude isotopes on; Dynamic exclusion 10s.

Supplementary Text S4: Preparation and characterization of CDs/protein

complexes

The extracted protein contents were quantified using Bicinchoninic Acid (BCA) Protein Assay Kit (CWBIO, China). The complex preparation was done by referring to the previous method with slight modifications.[24] The protein solution and the CDs solution were mixed in a 1:1 ratio.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) experimental operation is as follows. 100 mL of electrophoresis buffer (10×) was added to 900 mL of pure water for preparing electrophoresis buffer (1×). The configuration of 12% separating gel requires 4.56 mL pure water, 1.36 mL 30% Acrylamide-Bisacrylamide (Acr−Bis, 29:1), 2.0 mL sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) Stacking Gel Buffer (4×), 0.08 mL 10% ammonium persulfate (APS) and 0.008 mL N, N, N', N'-Tetramethylethylenediamine (TEMED), 5%. The configuration of the stacking gel requires 5.25 mL of pure water, 6.0 mL of 30% Acr−Bis (29:1), 3.75 mL of SDS−PAGE Separating Gel Buffer (4×), 0.15 mL of 10% APS and 0.006 mL of TEMED. The release gel is poured into the mold, and a small amount of pure water is used for sealing. Once the separating gel is polymerized, the pure water is removed, the stacking gel is poured into the mold and the comb is inserted. After the stacking gel polymerized, the comb was carefully removed. The electrophoresis sample preparation procedure is as follows. First, a sample solution containing 50 mg of protein is required to be prepared; Next, the loading buffer was added to the sample, and the mixture was reacted at 100 °C for 5 minutes; Finally, the mixture was centrifuged, and the supernatant was added to the sample well for electrophoresis. After the end, it was stained with Coomassie brilliant blue and imaged with a gel electrophoresis imager (BIO−RAD, Gel Doc XR+ and ChemicDoc XRS+ Imaging System, Version 6.0).

The TEM sample was prepared by the solution dispersion−dropping method, and the protein/CDs complex prepared in advance was ultrasonicated for 30 minutes in ice water and dropped on the copper grid (T10023, TILD), then used Talos F200X G2 electron microscope (FEI, USA) after drying to acquire a TEM image. The circular dichroism spectrum test was carried out according to the following steps. 5 mL of 0.5

$mg \cdot mL^{-1}$ protein and 5 mL of protein/CDs complex were used for this test, and 5 mL of blank solvent was used as a control. The sample was installed in a 1 mm quartz tube, and the circular dichroism spectrometer (J815, JASCO, Japan) with a scanning speed of 50 $nm \cdot min^{-1}$ was used to complete the test. To obtain accurate data, circular dichroism spectroscopy was performed at least three times. The isothermal titration calorimeter (ITC, TA, the United States) test is as follows. 2 mL of 1 $mg \cdot mL^{-1}$ CDs solution and 2 mL of 1 $mg \cdot mL^{-1}$ protein solution was used as the test sample solution, and the test mode was that CDs were dropped into the protein solution. The initial volume of CDs titration was 50 μL, and the starting volume of the protein was 350 μL. There are 25 drops of experiments, 2.02 μL per drop, 180 s interval, temperature of 25 ℃, stirring speed 350 $rpm \cdot min^{-1}$, temperature of 25 ℃, and CDs drip onto protein extract solution as a blank control. X−ray photoelectron spectrometer (XPS, Nexsa, Thermo Kalpha, USA) characterization were conducted by scientific compass test service platform.

Supplementary Text S5. Economic implication

Generally, lettuce is planted at a density of about 7.5×10^4 plants per hectare. The cost of CDs is \$0.07/g and the total dosage of each plant in the full life cycle is 0.125 mg based on the best performance concentration (5 $mg \cdot L^{-1}$). Thus, the additional cost of applying CDs to lettuce may be \$2.81 per hectare. A total yield of lettuce can be calculated about 30 t/hectare. Cold could cause 25% of the production loss, and economic losses can be up to $\$4.6\times10^3$ per hectare. It is predicted that the application of CDs could decrease lettuce yield loss to 20%, and the economic loss will be reduced by approximately $\$0.9\times10^3$ (the potential labor cost was not considered for CDs application).

Table S1. Significance of lettuce leaf metabolites in Non−CK and CDs under cold stress.

Classification	Name	Non-CK/CK		CDs/CK	
		FC	VIP	FC	VIP
Organic acids and their derivatives	Acrylic acid	1.042	0.023	0.647	1.259
	Asparagine	6.594	1.999	8.915	1.498
	Chicoric acid	0.523	1.481	0.550	1.086
	D-(-)-Glutamine	2.381	1.750	2.906	1.372
	D-(+)-Proline	1.615	1.376	1.646	1.434
	DL-Arginine	2.236	1.427	1.914	1.262
	DL-Glutamine	2.736	1.527	3.312	1.431
	DL-Lactic Acid	0.741	0.890	1.948	1.202
	DL-Malic acid	0.817	0.623	1.375	0.914
	DL-Norleucine	2.385	1.836	1.473	1.292
	Fumaric acid	0.930	0.381	1.484	1.116
	Jasmonic acid	1.505	1.029	2.958	1.448
	L-Aspartic acid	1.441	0.799	2.420	1.419
	L-Glutamic acid	1.367	0.731	1.875	1.430
	L-Phenylalanine	2.112	1.812	1.673	1.487
	L-Pyroglutamic acid	1.915	1.543	2.134	1.319
	L-Threonine	2.308	1.663	2.648	1.458
	Malonic acid	0.616	0.942	1.345	0.882
	Methylmalonic acid	1.019	0.144	0.601	1.452
	N-Acetyl-DL-glutamic acid	1.438	0.898	3.369	1.461
	N-Acetyl-L-phenylalanine	0.963	0.355	0.802	1.166
	Pipecolic acid	1.848	1.395	0.563	1.427
	Prolylleucine	0.738	0.934	0.831	1.165
	Salicylic acid	2.175	1.360	2.418	1.161
	Valine	2.157	1.749	1.599	1.301
Lipids and lipidoid molecules	(±)9-HpODE	0.923	0.579	0.421	1.426
	12-Oxo phytodienoic acid	0.867	0.673	0.304	1.417
	16-Hydroxyhexadecanoic acid	1.403	1.232	0.296	1.490
	1-Linoleoyl glycerol	0.534	1.756	0.765	0.894
	2-Hydroxycaproic acid	1.345	1.108	1.209	1.099
	2-Isopropylmalic acid	1.243	0.653	1.354	1.248
	3-Methyladipic acid	1.079	0.145	0.744	1.434
	9-HOTE	1.122	0.228	0.243	1.487
	9-Oxo-ODE	0.561	1.601	0.559	1.305
	Carvone	0.982	0.206	0.747	0.908
	Citral	0.494	1.638	0.462	1.224
	Corchorifatty acid F	0.770	1.088	0.301	1.502
	Cuminaldehyde	0.750	1.153	0.783	1.249
	Cynaroside	1.210	0.709	1.234	1.032
	D,L-Camphor	0.718	1.128	0.716	1.288

	Dihydroroseoside	0.765	1.080	0.828	1.023
	Mesterolone	1.043	0.040	0.287	1.497
	Neochlorogenic acid	0.927	0.528	0.471	1.448
Phenylpropanes and polyketides	7-Hydroxycoumarine	0.633	1.511	0.657	1.053
	Chalcone	0.630	1.487	0.058	1.536
	Eriodictyol	0.267	1.977	0.534	1.393
	Esculin	0.729	1.225	0.800	1.108
	Flavanone	0.606	1.124	0.022	1.491
	Miquelianin	0.743	1.323	0.792	1.189
	Naringenin	0.627	1.455	0.654	1.252
	Nictoflorin	0.585	1.613	0.651	1.350
	Phloretin	0.482	1.802	0.526	1.404
	Quercetin	0.658	1.514	0.739	1.175
	Quercetin-3β-D-glucoside	0.677	1.264	0.678	0.934
Organic heterocyclic compounds	2-Methoxy-9H-xanthen-9-one	0.799	1.159	0.049	1.522
	Adenine	0.979	0.310	0.650	1.226
	Caffeine	1.018	0.323	1.144	0.872
	DL-Tryptophan	1.260	0.803	0.596	1.310
	Indole-3-acrylic acid	1.227	0.714	0.597	1.314
	Oxepanone	0.851	0.631	0.291	1.463
	Pyraclostrobin	0.068	2.034	0.076	1.526
	Sedanolide	0.688	1.310	0.430	1.465
	β-Lapachone	0.665	1.238	0.024	1.554
Organic oxygen compounds	4,5-Dicaffeoylquinic acid	0.923	0.563	0.390	1.479
	Apocynin	1.065	0.173	0.820	1.017
	L-(+)-Tartaric acid	0.659	1.478	0.873	0.932
	Sucrose	0.662	1.253	1.210	1.140
	Triethylene glycol monobutyl ether	8.561	0.904	3.776	1.161
	α,α-Trehalose	0.462	1.664	1.174	1.263
Benzenes	1-Naphthol	0.467	1.751	0.749	0.900
	2,4-Dihydroxybenzoic acid	0.533	1.686	0.080	1.556
	2,5-di-tert-Butylhydroquinone	0.976	0.136	3.354	1.091
	2-Naphthalenesulfonic acid	1.003	0.053	1.392	1.077
	Eugenol	1.909	1.609	0.336	1.471
	Syringic acid	1.580	1.226	0.576	1.082
Organic nitrogen compounds	2-Amino-1,3,4-octadecanetriol	0.911	0.701	0.807	1.172
	2-Amino-1,3-octadecanediol	0.971	0.248	1.259	1.168
	Imidacloprid	0.918	0.075	0.339	1.077
	Oleoyl ethylamide	0.770	1.400	1.536	0.935
	α-Linolenoyl ethanolamide	3.348	1.507	0.356	1.453
Alkaloids and their derivatives	Hydromorphone	1.005	0.148	0.075	1.550
	Trigonelline	1.164	0.189	1.655	1.220
Nucleosides, nucleotides and	Uridine 5'-diphosphogalactose	1.987	1.373	2.217	1.383
	Xanthosine	3.123	1.719	0.434	1.367

analogs					
	13,14-dihydro-15-keto-tetranor Prostaglandin F1?	1.995	1.423	0.062	1.547
	1-Palmitoylglycerol	0.924	0.523	1.161	0.896
	1-Tetradecylamine	0.951	0.368	1.190	0.983
	2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3,4-dihydro-2H-1-benzopyran-4-one	0.276	1.985	0.512	1.500
	2-(3,4-Dihydroxyphenyl)-5-hydroxy-4-oxo-4H-chromen-7-yl 6-O-(6-deoxy-alpha-L-mannopyranosyl)-beta-D-glucopyranoside	0.503	1.572	0.592	1.462
	2-(hydroxymethyl)-6-[(E)-4-(1,2,4-trihydroxy-2,6,6-trimethylcyclohexyl)but-3-en-2-yl]oxyoxane-3,4,5-triol	0.759	1.262	0.663	1.438
	2-[3,8-Dihydroxy-8-(hydroxymethyl)-3-methyl-2-oxodecahydro-5-azulenyl]-2-propanyl hexopyranoside	1.120	0.325	0.181	1.542
	3-(4-Methylbenzoyl)acrylic acid	0.518	1.703	0.767	0.985
	3-[3-(beta-D-Glucopyranosyloxy)-2-hydroxyphenyl]propanoic acid	0.552	1.573	0.641	1.310
	3-{[(2R,3S,4S,5R,6S)-6-{[2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4H-chromen-3-yl]oxy}-3,4,5-trihydroxyoxan-2-yl]methoxy}-3-oxopropanoic acid	0.713	1.487	0.785	1.203
	4,4'-Bis(diethylamino)benzophenone	1.129	0.420	0.203	1.217
	4-methoxy-6-(prop-2-en-1-yl)-2H-1,3-benzodioxole	0.798	0.796	0.572	1.292
	4-methoxy-6-[2-(4-methoxyphenyl)ethyl]-2H-pyran-2-one	0.967	0.283	0.535	1.430
Others	5-(4-Carboxy-3-methylbutyl)-1,4a-dimethyl-6-methylenedecahydro-1-naphthalenecarboxylic acid	0.995	0.271	0.045	1.549
	5-(6-hydroxy-6-methyloctyl)-2,5-dihydrofuran-2-one	0.859	0.783	0.826	0.892
	5-(sec-butyl)-2-hydroxybenzaldehyde N-phenylhydrazone	1.019	0.062	0.305	1.175
	9-Oxo-10(E),12(E)-octadecadienoic acid	0.770	1.637	0.451	1.528
	Ageratriol	1.289	0.439	0.605	1.022
	Cyclopentylacetic acid	0.775	1.146	0.196	1.554
	Ethyl violet	2.816	0.781	0.434	1.065
	Glycerophospho-N-palmitoyl ethanolamine	1.365	0.752	0.485	1.473
	Luvangetin	0.699	1.171	0.057	1.537
	methyl 4-methyl-2-oxo-2H-pyran-6-carboxylate	0.866	0.559	0.287	1.496
	Michler's ketone	1.626	0.735	0.220	1.227
	N-(9-oxodecyl)acetamide	1.281	0.475	3.071	1.532
	N,N'-Diphenylguanidine	1.938	0.876	1.592	1.245
	N-Methyldioctylamine	1.600	0.445	1.239	1.161
	n-Pentyl isopentyl phthalate	1.247	0.208	0.426	1.317
	Shogaol	1.735	1.257	0.745	1.148
	Sorbicillin	0.875	0.702	0.831	0.975
	YNH	1.069	0.109	0.171	1.520
	α-Lapachone	0.792	0.880	0.125	1.535

FC: fold change

Non−CK/CK: Changes in natural growth state compared to cold stress

CDs/CK: Changes after application of CDs compared to cold stress

Table S2. Thermodynamic parameters of CDs and protein binding

	ΔH (kJ·mol^{-1})	ΔS (J·(mol·K)$^{-1}$)
Site 1	-273.6	-898.5
Site 2	2718	913.7

Table S3. Primer names and sequences used for qRT–PCR

Primer name	Sequences (5’ to 3’)
CBF 8−F	CATGTCGTAGCAAAAGCTCAGTCTTG
CBF 8−R	TTTCGTGAAGCCAACATCACTTCGG
CBF 12−F	TTGCAGCACAACCTCTGTCTTGAATG'
CBF 12−R	TCCCCTGTACACCGGATGTCTAGTCT
Cor 413−F	TGTTCGTCGCTCGCTCAC
Cor 413−R	ACCCTCGTTTCCATTTCTGC

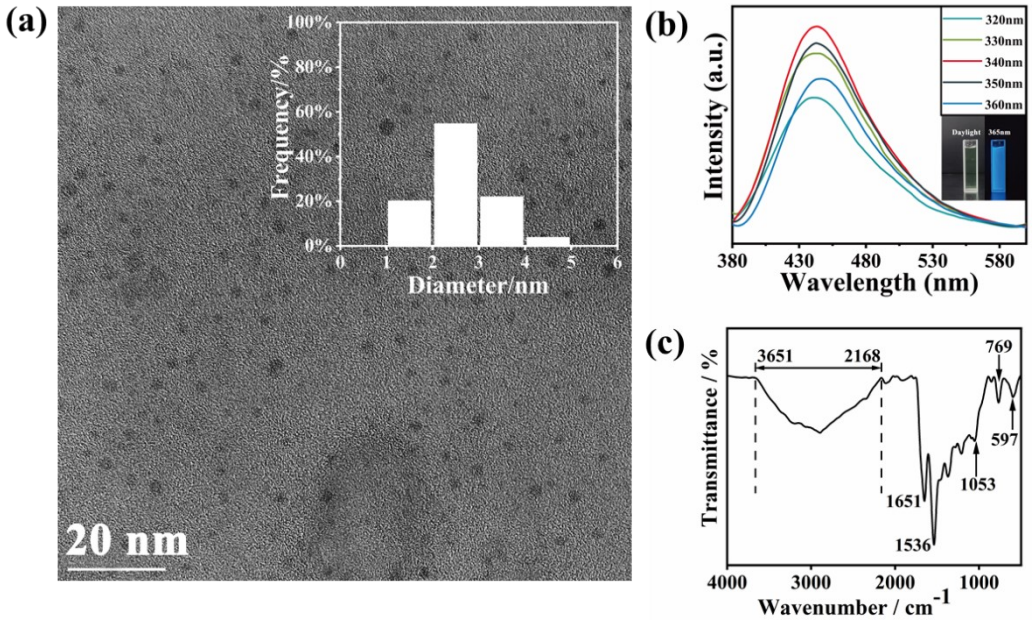

Fig. S1. Characterization of CDs. (a) TEM image of CDs. (b) PL spectra of CDs. (c) FTIR spectrum of CDs.

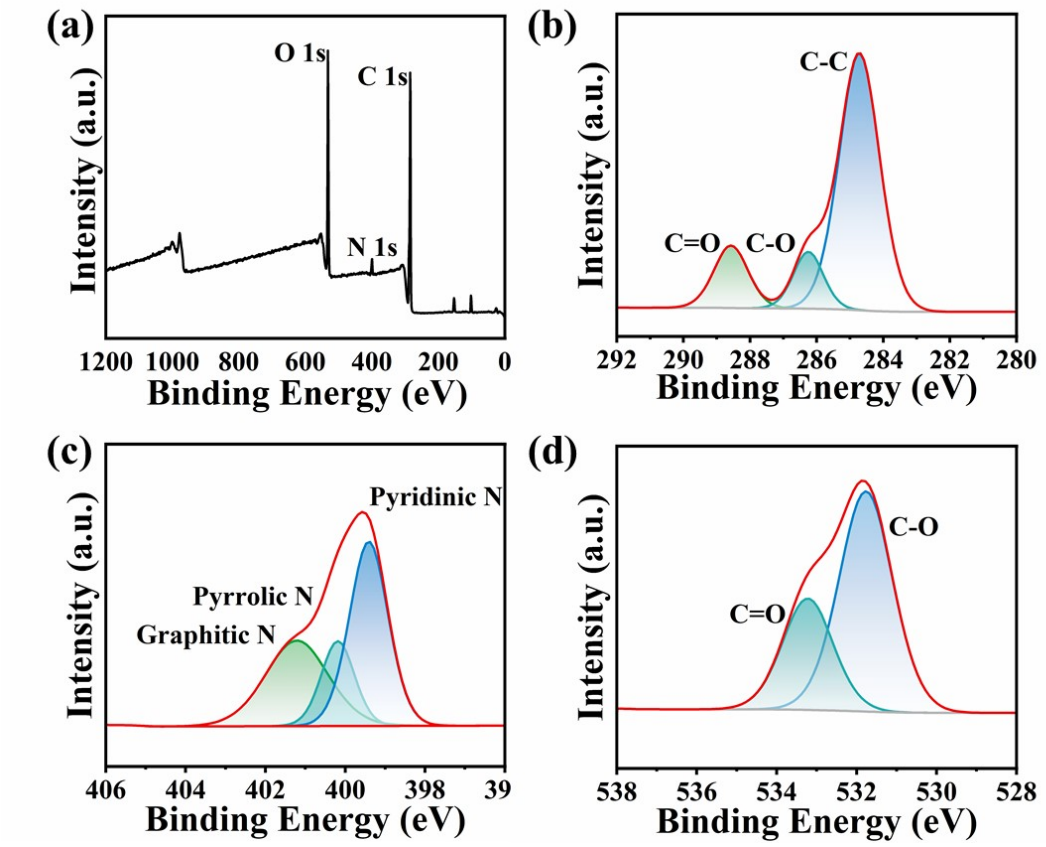

Fig. S2. XPS spectra of CDs. (a) XPS full spectrum of CDs. (b) High–resolution C 1s XPS spectrum. (c) High−resolution N 1s XPS spectrum. (d) High-resolution O 1s XPS spectrum.

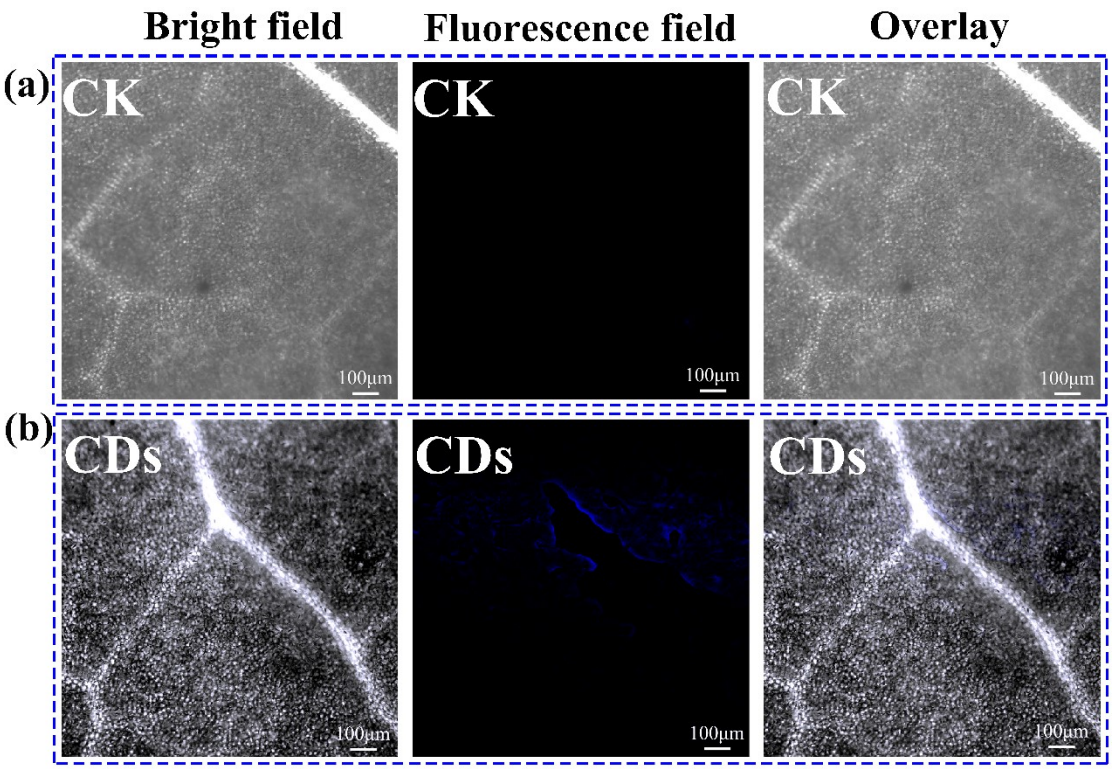

Fig. S3. Confocal images of CDs in lettuce leaves. (a) CK, under natural growth conditions; (b) CDs, observed after spraying CDs.

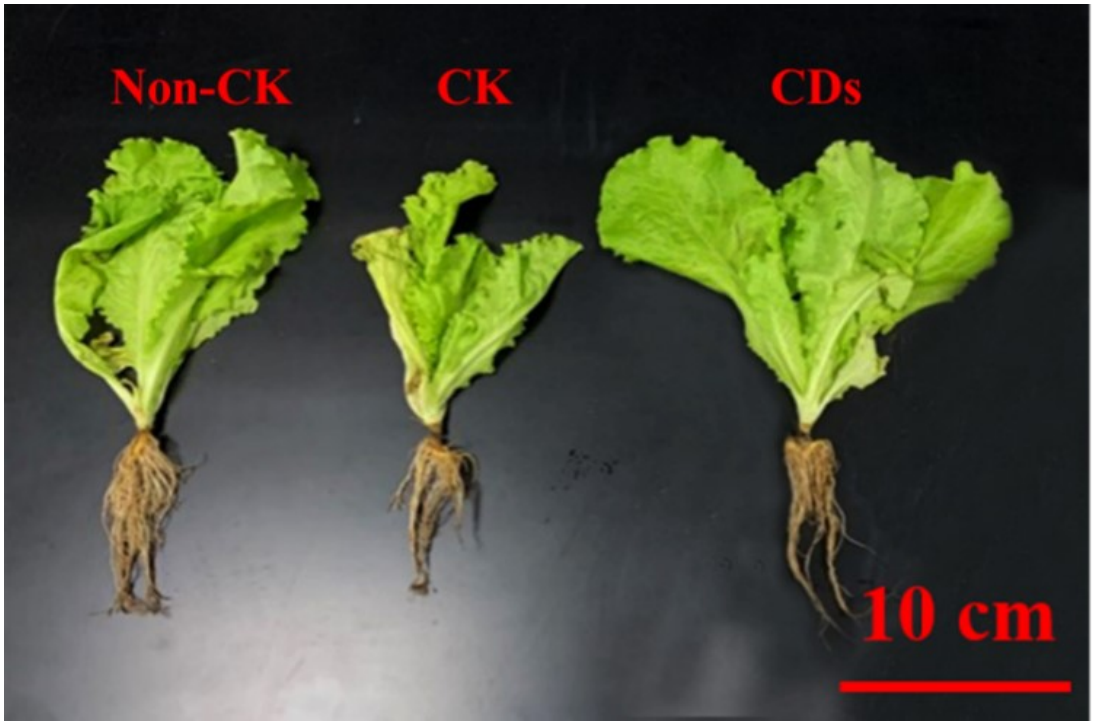

Fig. S4. Photos of lettuce. The lettuce was collected and photographed on the 56th day. The Non-CK group was not treated, and it was grown naturally; the CK group was naturally grown to the 55th day, and placed in an environment at 4°C for 12 hours; the

CDs group were sprayed CDs continuously for 5 days, and placed in a 4°C environment for 12 hours.

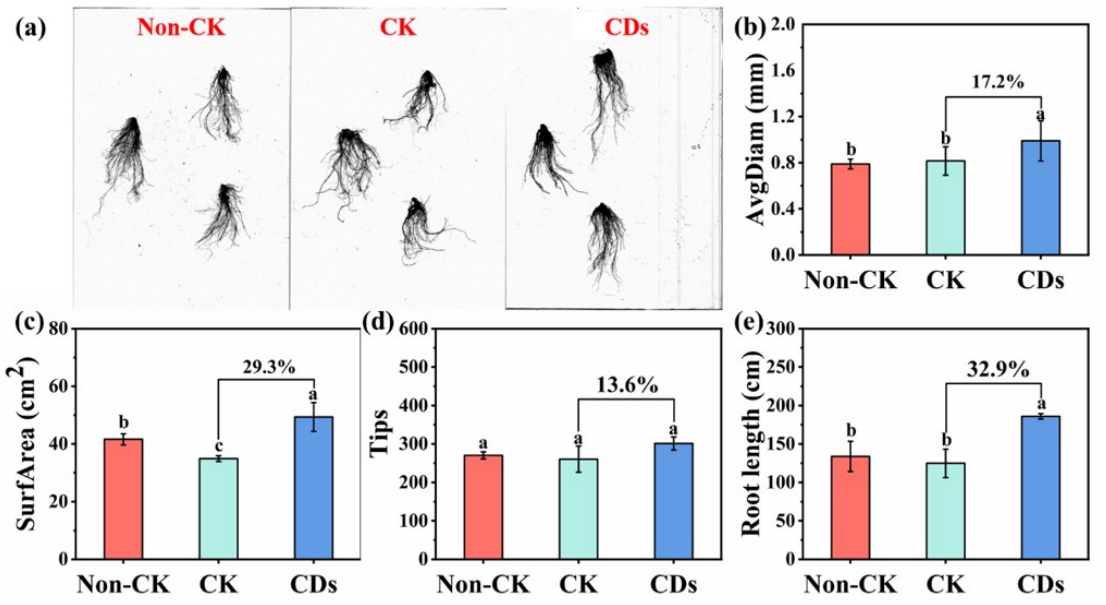

Fig. S5. Photos and parameters of lettuce root. (a) Photo of the root effect of lettuce. (b) Average root diameter of lettuce roots. (c) The surface area of the lettuce root. (d) Number of root tips of lettuce roots. (e) Root length of lettuce root.

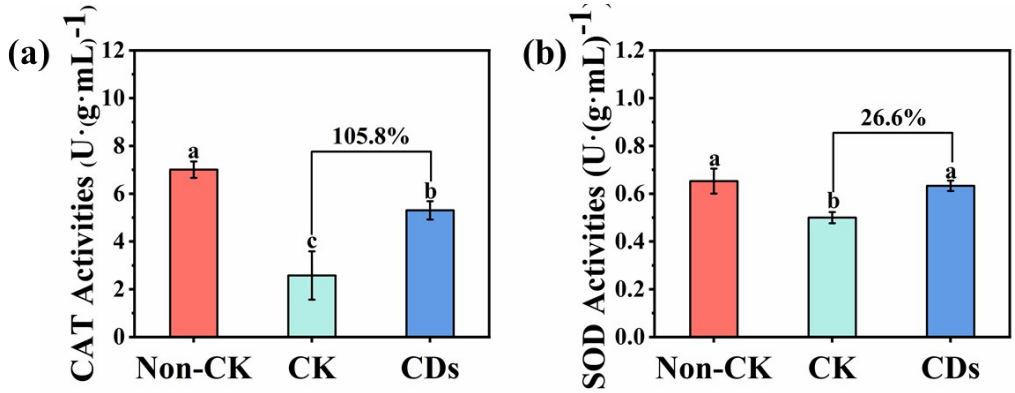

Fig. S6. Enzyme activity of antioxidant system in lettuce leaves. (a) CAT activity. (b) SOD enzyme activity. (p<0.05).

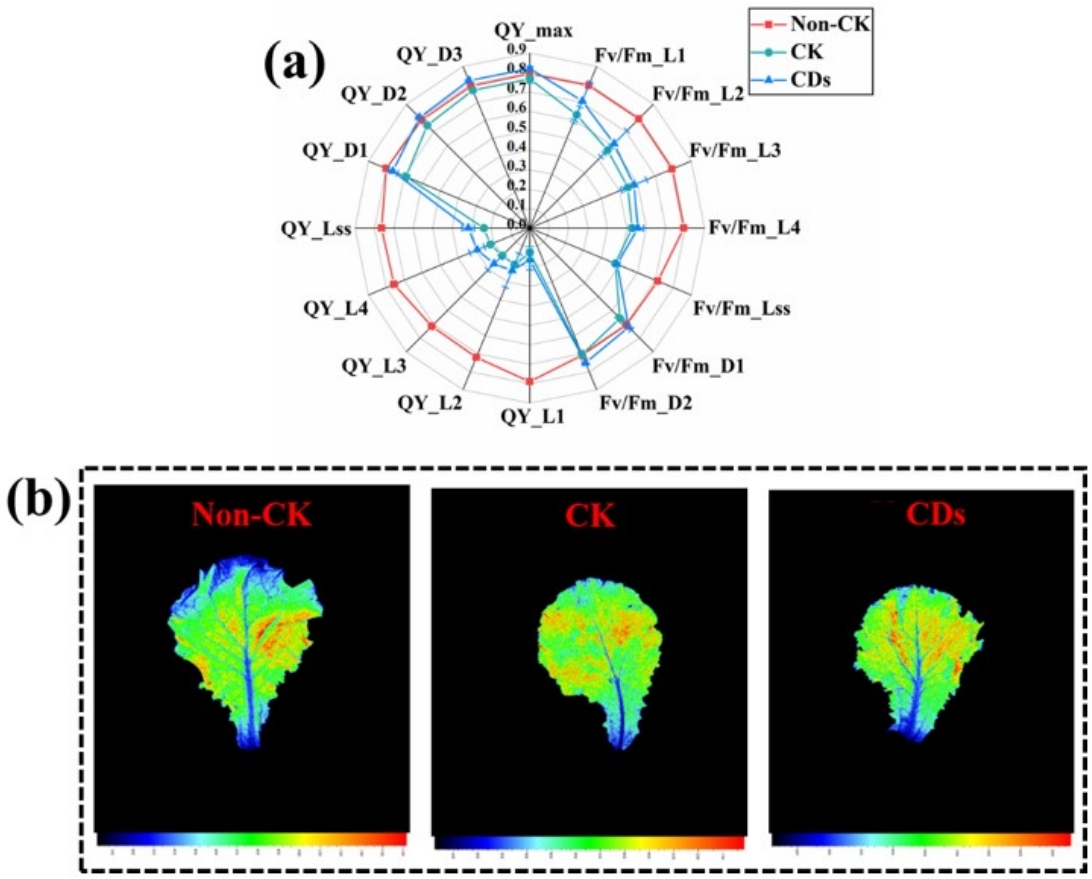

Fig. S7. Chlorophyll fluorescence parameters and effect photos of lettuce**.** (a) Chlorophyll fluorescence parameters; (b) Chlorophyll fluorescence image.

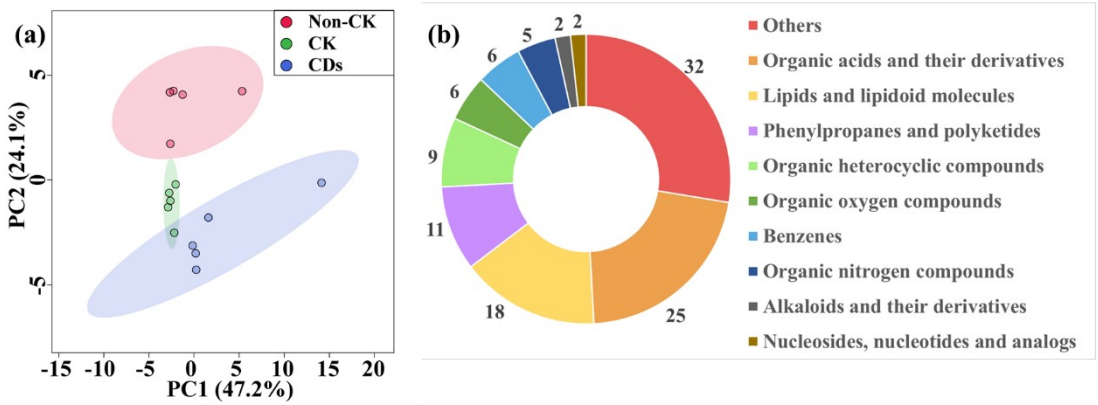

Fig. S8. CDs regulate the metabolism of lettuce leaves. (a) Principal component analysis (PCA) diagrams of metabolites in lettuce leaves in different groups (Non-CK, CK and 5 mg·kg^{-1} CDs). (b) Classification of significantly different metabolites in lettuce leaves.

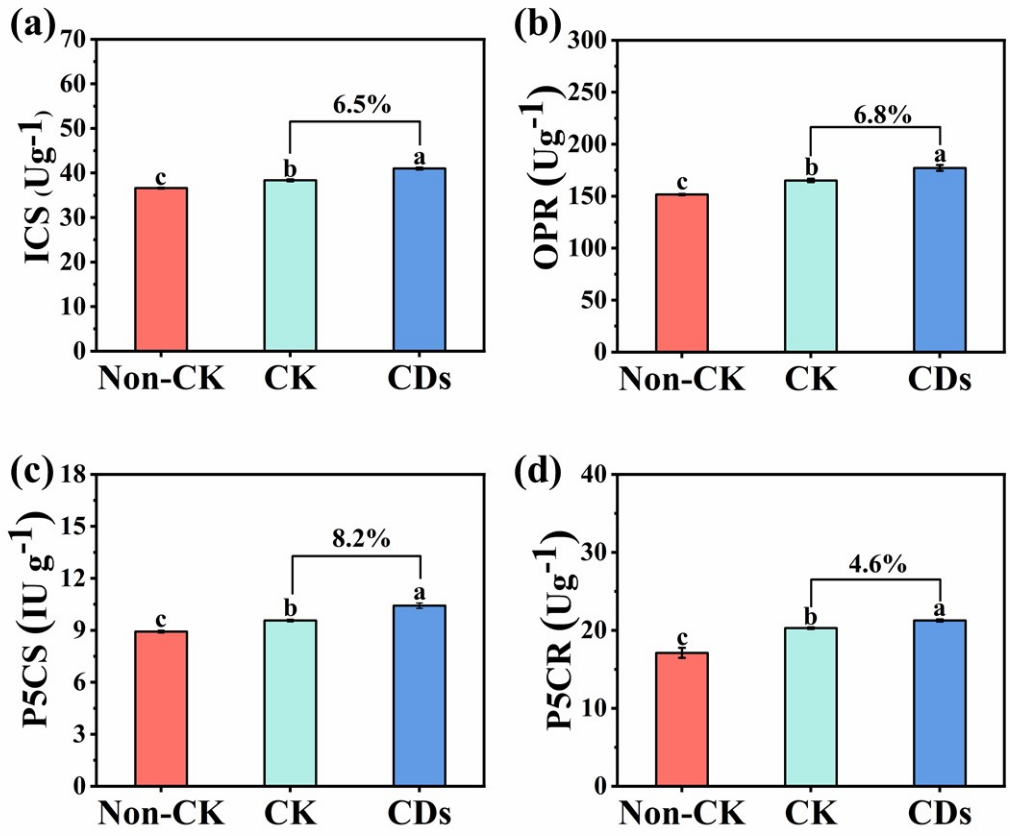

Fig. S9. Rate-limiting enzyme activity of related metabolites. (a) Rate-limiting enzyme activity of salicylic acid synthesis; (b) rate-limiting enzyme activity of jasmonic acid synthesis; (c) and (d) rate-limiting enzyme activity of proline synthesis.

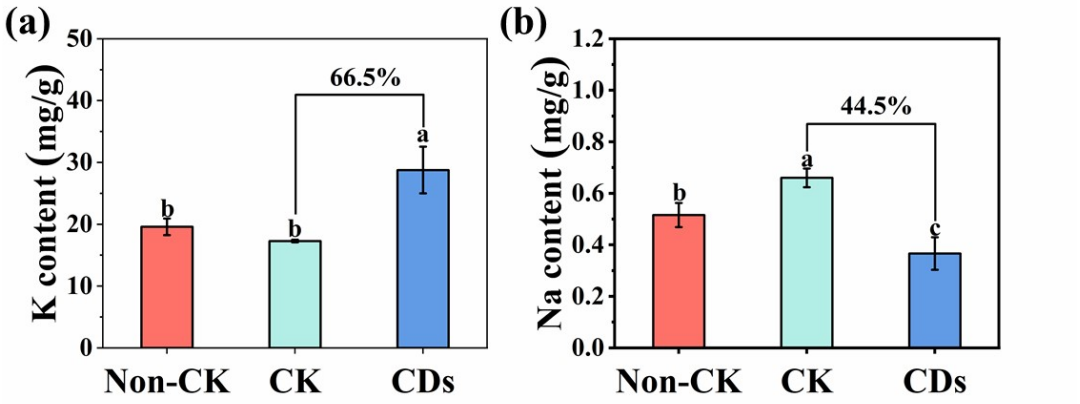

Fig. S10. Maintaining osmotic balance in lettuce cells. (a) K^+ content in lettuce cells. (b) Na^+ content in lettuce cells.

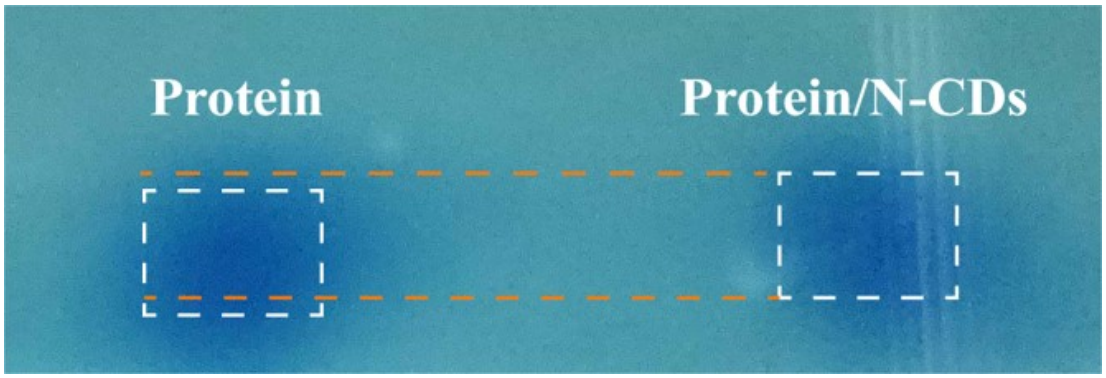

Fig. S11. SDS-PAGE images of proteins, CDs/proteins. The framed area represents the region of major movement during electrophoresis.

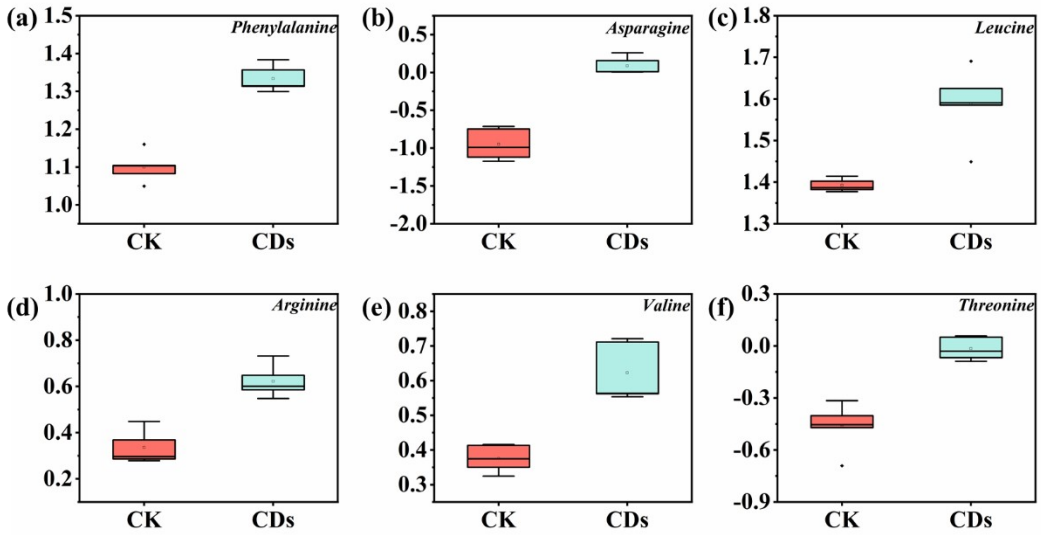

Fig. S12. Spraying CDs changes the contents of related amino acids in lettuce leaves. (a)-(f) are phenylalanine, asparagine, leucine, threonine, valine, and arginine, respectively.

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